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Analysis of Linear Epitopes Recognised by the Primary Human Antibody Response to a Variable Region of the Attachment (G) Protein of Respiratory Syncytial Virus

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The sites of linear epitopes in a variable region of the attachment (G) glycoprotein of respiratory syncytial virus (RSV) that are recognised by the human antibody response were examined. Two sets of overlapping 12mer peptides each representing the carboxy-terminal 84 or 85 amino acids of the G protein of two group A isolates of human RSV were synthesised. These peptides were analysed using enzyme-linked immunosorbant assays (ELISA) for their reactions with sera obtained from infants with primary RSV infection. Four pairs of overlapping peptides were found to react variously with the sera, the reactions depending on the infecting genotype of RSV. Further 9mer peptides based on natural variants in the epitope areas were then synthesised to determine the specificity of the human antibody response and it was found that single amino acid changes could abrogate recognition by these polyclonal sera. All the linear epitopes found are involved in potential N-glycosylation sites in at least some isolates of RSV. ***J. Med. Virol. 51:297–304, 1997.***

KEY WORDS: linear epitopes; respiratory syncytial virus; G glycoprotein

INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract disease in infants and vulnerable adults. RSV is unusual in that it can frequently reinfect individuals and infects babies despite the presence of maternal antibody. Isolates of RSV can be divided into two groups, A and B, on the basis of their reactions with monoclonal antibodies and by nucleotide sequencing [Anderson et al., 1985; Mufson et al., 1985; Gimenez et al., 1986; Johnson and Collins, 1989; Cane and Pringle, 1991]. Each of the groups can be divided further into a number of genotypes, again using nucleotide sequencing and to a lesser extent, monoclonal antibodies [Cane and Pringle, 1991, 1992, 1995; Garcia et al., 1994]. The gene that shows the greatest variability both within and between the groups is that coding for the attachment (G) protein, with isolates from the two groups varying by 47% in amino acid sequence [Johnson et al., 1987] and within the groups by up to 20% amino acid sequence for this protein [Cane et al., 1991; Sullender et al., 1991].

The G protein of human RSV is between 289 and 299 amino acids in length depending on the strain and is oriented in the membrane with the carboxy-terminal three fourths extracellular. The protein is heavily glycosylated with both N and O linked sugars and is rich in serine, proline, and threonine: it is thus unusual for a viral glycoprotein and appears more similar to cellular mucinous proteins [Wertz et al., 1985; reviewed by Sullender and Wertz, 1991].

The variable regions of the protein lie in two parts of the ectodomain separated by a central highly conserved region [Johnson et al., 1987; Cane et al., 1991; Sullender et al., 1991]. Analysis of escape mutants resistant to murine monclonal antibodies which show variable reactions with RSV isolates showed that most of the mutants had amino acid changes in the variable carboxy-terminal region of the protein indicating that this region includes antigenic areas [Garcia-Barreno et al., 1990; Rueda et al., 1991]. However, competition enzyme-linked immunosorbant assays (ELISA) between human convalescent sera and murine monoclonal antibodies failed to locate any reaction of the human sera to this region [Palomo et al., 1991].

There is some evidence that there is progressive accumulation of amino acid changes in the variable regions of the G protein of group A isolates [Cane and Pringle, 1995]. Currently circulating group A isolates

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can be divided into a number of genotypes designated SHL1/3/4, SHL2, SHL5, and SHL6 [Cane and Pringle, 1992]. It has recently been demonstrated that there is an antibody response to the carboxy-terminal region of the G protein during primary infection and that this response can be highly specific to the infecting genotype of virus [Cane et al., 1996]. The experiments reported now were carried out to determine whether there are linear epitopes in this region that can be detected by the reaction of sera from infants experiencing their first infection with RSV, with peptides based on the amino acid sequence of the G protein.

This approach has been used before: Norrby et al. [1987] looked at the reaction of human convalescent sera, murine monoclonal antibodies, and hyperimmune rabbit sera with overlapping 15mer peptides based on the amino acid sequence of the ectodomain of the G protein of the prototype group A strain, A2. They found that human convalescent sera reacted only with their peptides 11, 12, and 15, corresponding to amino acids 184–198, 174–188, and 144–158 respectively. Peptides 11 and 12 lie in a highly conserved region of the protein, and peptide 15 in a fairly conserved region. These authors did not find any reaction of the human sera with the long comparatively hydrophilic carboxy-terminal part of G and thought that this region might not be immunogenic. However, the work was done before it was appreciated that there was considerable variability in this region of the G protein. In addition, Langedijk et al. [1996] carried out a similar pepscan analysis of bovine RSV G protein using polyclonal sera and again found antibody reactions only with the central conserved region of the protein: this study also used only prototype BRSV sequence for design of peptides.

The results described now show that there are indeed linear epitopes recognised by the human antibody response, present in the carboxy-terminal region of the G protein, but that their recognition is highly dependent both on the amino acid sequences of the peptides used as targets in the tests, and on the infecting genotype of virus.

MATERIALS AND METHODS

Peptide Synthesis

Non-cleavable peptides were synthesised on polypropylene gears on stems using a multipin peptide synthesis kit (Chiron Mimotopes Peptide Systems, Clayton, Victoria, Australia), using Fmoc amino acids with the terminal amino group capped by acetylation. Firstly, two sets of peptides were synthesised each corresponding to the carboxy terminal 84 or 85 amino acids (i.e., 214–297/8) of the G protein of virus isolates RSB89-6256 and RSB89-6190 respectively [Cane et al., 1991]. The genotypes of current group A isolates have been designated SHL1-6, although SHL1, 3, and 4 have been found to have very similar G gene sequences so are not distinguished in this study [Cane and Pringle, 1992; Cane et al., 1996]. RSB89-6256 is genotype SHL3 and RSB89-6190 is genotype SHL2. Sequences from these genotypes were chosen for the initial peptide synthesis as most of the human sera available (see below) came from babies infected with these genotypes of RSV. The first set of peptides synthesised were 12mers, each overlapping by three amino acids, as illustrated in Figure 1. In the case of peptides based on the amino acid sequence of RSB89-6190, the carboxy-terminal 12mer overlapped by one amino acid only. Initially, a total of 51 RSV specific peptides were synthesised, together with two control peptides suggested by the Multipin kit manufacturers (PLAQGGGGGGGG and GLAQGGGGGGGG). Subsequently, a further set of 9mer peptides based on the natural variants of amino acids 250–258, 265–273, and 283–291 was also synthesised. These peptides are listed in Table I.

Sera

The patients from which the primary infection sera were obtained have been described previously [Cane et al., 1996]. These sera were made available through the generosity of Dr. H. Thomas, Southmead Hospital, Bristol, UK. Only sera that had been shown previously to react with the terminal 85/86 amino acids of the G protein as expressed as GST fusion proteins in *E. coli* were selected for this study [Cane et al., 1996]. A monoclonal antibody specific for the carboxy-terminal region of the G protein under study in this report, 021/9G [Garcia et al., 1994], was kindly provided by Dr. J.A. Melero, Instituto de Salud Carlos III, Madrid, Spain.

Enzyme-Linked Immunosorbant Assay (ELISA)

All manipulations were carried out with the peptides still covalently linked to the pins. The pins carrying the peptides were blocked using 5% dried milk in PBS with 0.1% Tween 20 (PBS-T). All sera were reacted with the peptides at a dilution of 1:1000 in blocking buffer. After washing extensively with PBS-T, the pins were incubated with goat anti-human IgG (heavy and light chain) conjugated with horseradish peroxidase, washed extensively, and developed using *o*-phenylenediamine dihydrochloride. The reactions were stopped by removing the pins and then adding 2.5 M sulphuric acid.

Antibody was removed from the pins by sonication in 0.1 M phosphate buffer, pH 7.2 containing 1% SDS and 0.1% 2-mercaptoethanol at 60°C, followed by washing in hot water and hot methanol.

RESULTS

Peptide Synthesis

The positive control peptide, PLAQGGGGGGGG, reacted with the control monoclonal antibody supplied with the Multipin kit while the negative control peptide, GLAQGGGGGGGG, did not, as expected from the peptide synthesis kit protocol. In addition, 12mer peptides 8, 9, and 10 of both isolates reacted with monoclonal 021/9G, for which escape mutant changes have been mapped to amino acid 244 (R-S) [Martinez, 1996]. These results indicate that the peptide synthesis had proceeded satisfactorily. However, it is unlikely that all

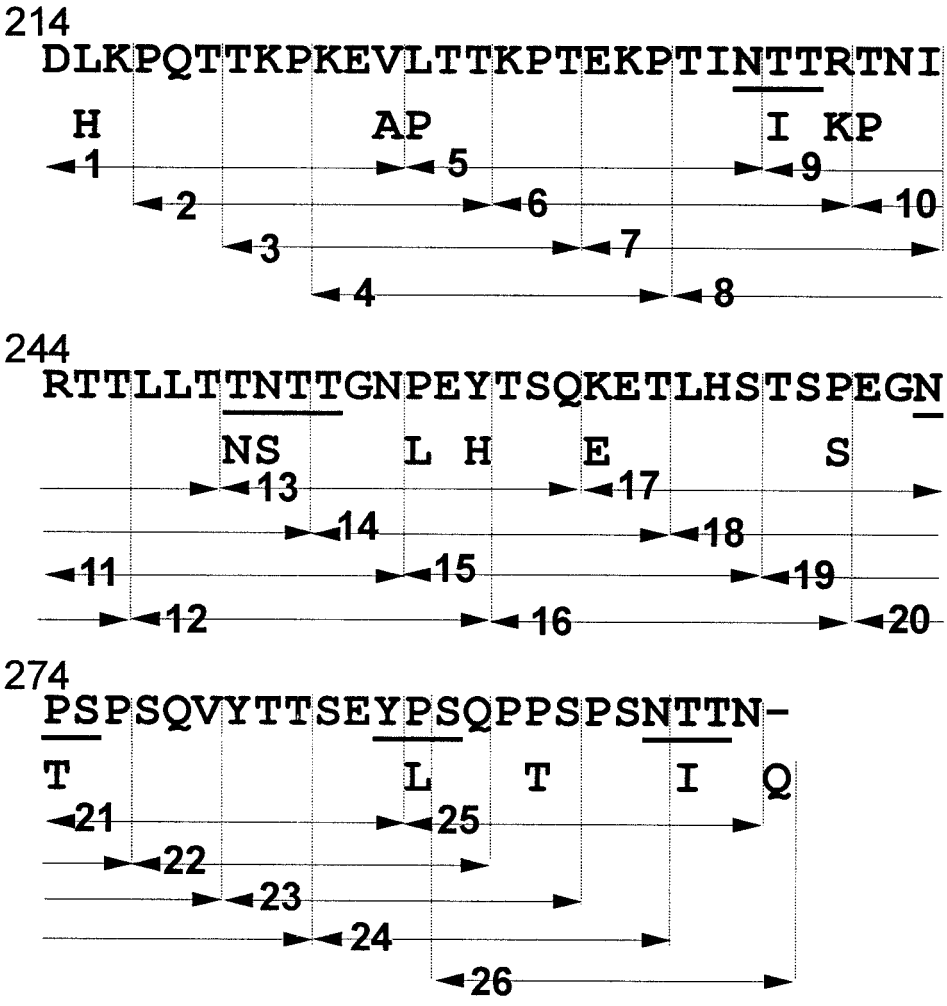

Fig. 1. Twelve amino acid long overlapping peptides representing the carboxy-terminal 84 or 85 amino acids of the G protein of RSV isolates RSB89-6256 or RSB89-6190 respectively [Cane et al., 1991]. Sequence given in the top line is that of RSB89-6256, with changes in RSB89-6190 shown in the lower line. Peptide 26 was synthesised for RSB89-6190 only. Positions of potential N-glycosylation sites in at least some RSV isolates (see text) are underlined.

the peptides were synthesised with the same efficiency so direct comparisons of degrees of reactions given by different peptides are difficult.

Primary Response to Overlapping 12mer Peptides Based on Amino Acids 214–298

Acute and convalescent primary RSV infection sera from five babies, together with convalescent sera only from a further five babies, were reacted with the 51 12mer peptides derived from the carboxy terminal 84/85 amino acids of two isolates (RSB89-6256 and RSB89-6190) of RSV. The peptides that the sera reacted with are summarised in Table II, and some typical results are illustrated in Figure 2. In most cases the ELISA tests were repeated for each of the sera and the results were very reproducible. However, it was not possible to repeat all assays particularly those involving the acute sera because of the extremely small quantities of sera available from most of the patients. In addition, again due to the scarcity of sera, only one antibody dilution (1:1000) was used.

The acute serum from baby 25 reacted with only one peptide: peptide 17 of RSB89-6190 (Fig. 2a). The convalescent serum from this baby showed no detectable change in reaction with this peptide, but displayed strong reactions with peptides 17 and 18 of RSB89-6256 and a weaker reaction with peptide 18 of RSB89-6190 (Fig. 2b). This baby was infected with genotype SHL6, which although having a distinct sequence over some of the area covered by the peptides, differs from RSB89-6256 only at position 270 (P-S) for peptides 17 and 18.

Baby 47 was infected with RSV genotype A:SHL1/3/4, i.e., with a strain very similar to RSB89-6256. The acute serum from this baby failed to react with any of the peptides (Fig. 2c), while the convalescent serum showed strong reactions with peptides 17 and 18 from both peptide series (Fig. 2d). The acute serum of baby 27, infected with this same genotype, reacted with peptide 17 of RSB89-6256, while the convalescent serum reacted strongly with peptides 17 and 18 from both isolates (data not shown).

TABLE I. 9mer Peptides Based on Natural Variants of G Protein Amino Acids 250–258, 265–273, and 283–291

Peptide number	Amino acids	Peptide sequence	Isolate	Reference
52	250–258	NSTTGNLEH	RSB89-6190	Cane et al., 1991
53		TNTTGNPEY	RSB89-6256	Cane et al., 1991
54		SNTTGNPEL	A2	Wertz et al., 1985
55		NNTTGNPKL	Long	Johnson et al., 1987
56		SNTTRNPEL	RSB89-1734	Cane et al., 1991
57		SNTARNPEL	RSB89-642	Cane et al., 1991
58		INTTGNPEH	RSB91-48	Cane and Pringle, 1995
59		TNTTGNPEH	Mad/1/89	Garcia et al., 1994
60		SNTTGNPEH	RSB94-6159	Unpublished
61		NSTTGNPEH	Mon/1/90	Garcia et al., 1994
62		SNTTGNLEH	Mad/1/88	Garcia et al., 1994
63		NSTTGNLEL	Mad/9/93	Garcia et al., 1994
64		NNTTGNPEH	RSS2	Cane and Pringle, 1995
65		NNTTGNPEL	RSSw23891	Cane and Pringle, 1995
66		SSTTGNLEH	RSC6079	Cane and Pringle, 1995
67		NNTTGYLEL	T2-4221	Unpublished
68		NNTTGNPEF	Ct8-7162	Unpublished
69		NNTTGNPEN	RSGam35	Unpublished
70	265–273	LHSTSPEGN	RSB89-6256	Cane et al., 1991
71		LHSTSSEGN	RSB89-6190	Cane et al., 1991
72		FHSTSSEGN	A2	Wertz et al., 1985
73		FHSTSSESN	RSG1503	Cane and Pringle, 1995
74		LHSTFSEGN	NC8870	Cane and Pringle, 1995
75		LHSTSSDGN	RSS2	Cane and Pringle, 1995
76		LHSTTSEGN	RSF50676	Cane and Pringle, 1995
77		YHSTSSEGN	RSG1545	Cane and Pringle, 1995
78	283–291	SEYPSQPPS	RSB89-6256	Cane et al., 1991
79		SEYPSQPSS	A2	Wertz et al., 1985
80		SEYPPQPSS	RSB89-642	Cane et al., 1991
81		SEYPSQPLS	RSGam66	Unpublished
82		SEYSSQPSS	RSG1504	Cane and Pringle, 1995
83		SEYQSQPPS	Mad/6/92	Garcia et al., 1994
84		SEHSSQPSS	NC8870	Cane and Pringle, 1995
85		SEYLSQPPS	RSB90-8336	Cane and Pringle, 1995
86		SEYLSQPTS	RSB89-6190	Cane et al., 1991
87		SEYLSQPSS	NC2825	Cane and Pringle, 1995
88		SEYLSQSPS	RSB90-8106	Cane and Pringle, 1995
89		SEYLSQTPS	H1414	Cane and Pringle, 1995
90		SEYLSQPLS	RSA32	Cane and Pringle, 1995
91		SENLSQSLS	RSB94-6519	Unpublished
92		SEYLSQSLS	Mon/2/88	Garcia et al., 1994
93		FEYLSQSPS	Mad/4/90	Garcia et al., 1994

Baby 109 was infected with RSV genotype A:SHL2. As with baby 47, the acute serum reacted with none of the peptides. The convalescent serum reacted with peptide 7 from isolate RSB89-6190 only and to a lesser extent with peptide 6 from the same isolate (data not shown).

Both acute and convalescent sera from baby 31 (infected with SHL1/3/4 genotype) serum reacted strongly with peptides 23 and 24, and to a lesser extent with peptide 22, of isolate RSB89-6256 only. The sera from this baby also showed some reactions with peptides 17 and 18 from both isolates (Fig. 2e,f). The reason for the lack of change between the acute and convalescent sera from this baby could be due to the acute serum being collected relatively late in the course of the infection.

Convalescent sera only from a further five infants were reacted with this first series of peptides. These sera showed similar reactions to those observed for the other babies as shown in Table II: some sera (babies 8 and 35) reacting with peptides 17 and 18 while others (165 and 495) reacting with peptides 6 and 7 (Fig. 2g,h). Serum from baby 706 also reacted with peptide 12 and 17 of strain RSB89-6256 and peptide 7 of strain RSB89-6190.

It was clear that small differences between the peptides could abrogate recognition so failure to detect a reaction with particular peptides could be due to differences in the sequence of the infecting strain of virus compared to the test sequences. The specificity of the reaction with the appropriate peptides was therefore examined further.

Specificity of Response to Peptides

A set of 42 9mer peptides was synthesised based on the naturally occurring variants of amino acids 250–258, 265–273, and 283–291 of the G protein of group A RSV isolates. These corresponded to the overlapping portions of peptides 12–13, 17–18, and 23–24 from the first series. Reactions with peptides 6–7 from the first series were not analysed further due to small numbers

TABLE II. Reactions of Baby Sera With 12mer and 9mer Peptides

Baby	Infecting genotype	12mer peptides reacting with acute serum	12mer peptides reacting with convalescent serum	9mer peptides reacting with convalescent serum
8	A:1/3/4	nd[a]	RSB89-6256: 17,18 RSB89-6190: 4,17,18	70, 71, 72, (73),[b] 75, 77
25	A:6	RSB 89-6256: none RSB 89-6190: 17	RSB89-6256: 17,18 RSB89-6190: 17, 18	70, 71, 72, 75, 77
27	A:1/3/4	RSB 89-6256: 17 RSB 89-6190: none	RSB89-6256: 17, 18 RSB89-6190: 17, 18	70, 71, 72, (73), 75, 77
31	A:1/3/4	RSB 89-6256: 17, 18, 22, 23, 24 RSB 89-6190: 17, (18)	RSB89-6256: 17, 18, 22, 23, 24 RSB89-6190: 17, 18	70, 71, 72, 75, 77, 78, 79, 80, 81, 82, (83)
35	A:nd	nd	RSB89-6256: 16, 17, 18 RSB89-6190: (17, 18)	70, (71), (72), 75, 77
47	A:1/3/4	RSB 89-6256: none RSB 89-6190: none	RSB89-6256: 17, 18 RSB89-6190: 17, 18	(52), 70, 71, 72, (73), 75, 77
109	A:2	RSB 89-6256: none RSB 89-6190: none	RSB89-6256: none RSB89-6190: (6), 7	none
165	A:nd	nd	RSB89-6256: (7) RSB89-6190: 6, 7	none
495	A:2	nd	RSB89-6190: (6), 7	none
706	A:2	nd	RSB89-6256: 12, 17 RSB89-6190: 7	58
E1	nd	nd	nd	55

[a]nd: Not determined.
[b]( ): brackets indicate weak reaction.

of sera reacting with these peptides. The peptide sequences and their derivation are shown in Table I. In all 18 different peptides were synthesised for amino acids 250–258, 8 for amino acids 265–273 and 16 for amino acids 283–291. The reactions of the baby sera with these peptides are shown in Table II.

As illustrated above the convalescent baby sera were variable as to which of the antigenic regions they recognised. Only two baby sera reacted clearly to peptides based on amino acids 250–258: Baby 706 serum reacted with peptide 58 together with weak reactions with peptides 70–73, 75, and 77 (Fig. 3a), while baby E1 reacted only with peptide 55 (data not shown). The sequence of peptide 58 differs from those of peptides 59, 60, and 64 at only one position, namely the isoleucine residue at 250 is substituted with threonine, serine, or asparagine respectively and these single substitutions are sufficient to completely abrogate recognition of these peptides by this individual's serum. The sequence of peptide 55 differs from that of 65 with the substitution of a lysine residue at position 257 with a glutamic acid residue.

Several of the baby sera reacted with some of the peptides derived from amino acids 265–273. Variations in residue 265 (F/L/Y) and 271 (D/E) had little effect on recognition by the infants' antibody, but change of residue 269 from serine to either threonine (peptide 76) or phenylalanine (peptide 74) completely abrogated recognition. Change of residue 272 from glycine to serine (peptide 73) reduced reaction with the sera considerably. The relative intensity of reactions with these peptides varied between the babies, with for example, baby 25's serum reacting markedly more strongly with peptide 70 than with peptides 75 and 77 (Fig. 3b) while baby 47's serum reacted equally strongly with peptides 70, 71, 72, 75, and 77 (Fig. 3c): these results were reproducible between experiments. Pooled sera from mice vaccinated with a vaccinia recombinant expressing the G protein from strain A2 reacted with peptides 72, 73, and 77 (data not shown), indicating that perhaps in this instance the critical residue may be amino acid 265 where change of the phenylalanine to leucine but not to tyrosine affected recognition.

Serum from only one baby (31) reacted with peptides derived from amino acids 283–291 (Fig. 2e,f). Baby 31's serum reacted strongly with peptides derived from both this region and from the 265–273 region. With respect to the 283–291 region, this baby's serum reacted strongly with peptides 78, 79, 80, 81, and 82, weakly with peptide 83 and failed to react with the other variant 283–291 peptides (Fig. 3d). The key amino acids in this epitope appear to be residues 286 and 287. The single change of residue 287 from proline to leucine abrogated all recognition. Change of this residue to serine had no effect unless the tyrosine residue was also changed to histidine in which case no reaction of the serum with the peptide occured. Change of the 287 proline residue to glutamine resulted in much reduced recognition.

DISCUSSION

The results demonstrate that there are specific antibody responses during primary RSV infection to linear epitopes in the carboxy-terminal region of the G protein. The sites recognised vary with the infecting genotype of virus: serum from babies infected with A:SHL2 reacted with peptides 6 and 7 (amino acids 229–243), while serum from babies infected with A:SHL1/3/4 reacted with peptides 17 and 18 (amino acids 262–276) and, in one case, peptides 23 and 24 (amino acids 280–294). Sera from two babies also reacted peptides from amino acids 247–258. It seems likely that these polyclonal sera are not all recognising identical epitopes

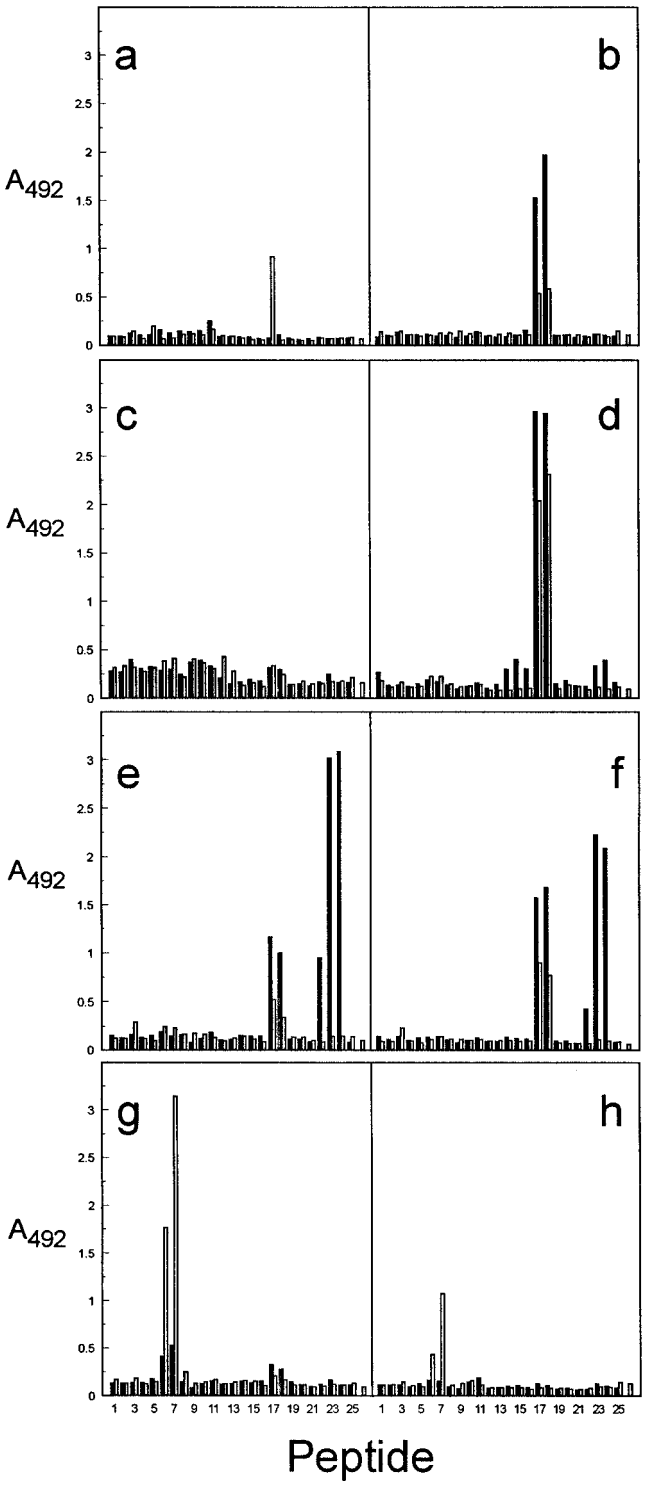

Fig. 2. Reactions of baby sera in ELISA tests with series of 12mer peptides illustrated in Figure 1. **a:** Baby 25 acute serum; **b:** baby 25 convalescent serum; **c:** baby 47 acute serum; **d:** baby 47 convalescent serum; **e:** baby 31 acute serum; **f:** baby 31 convalescent serum; **g:** baby 165 convalescent serum; **h:** baby 495 convalescent serum. Filled bars are data from peptide series based on isolate RSB89-6256; empty bars are data from series based on isolate RSB89-6190.

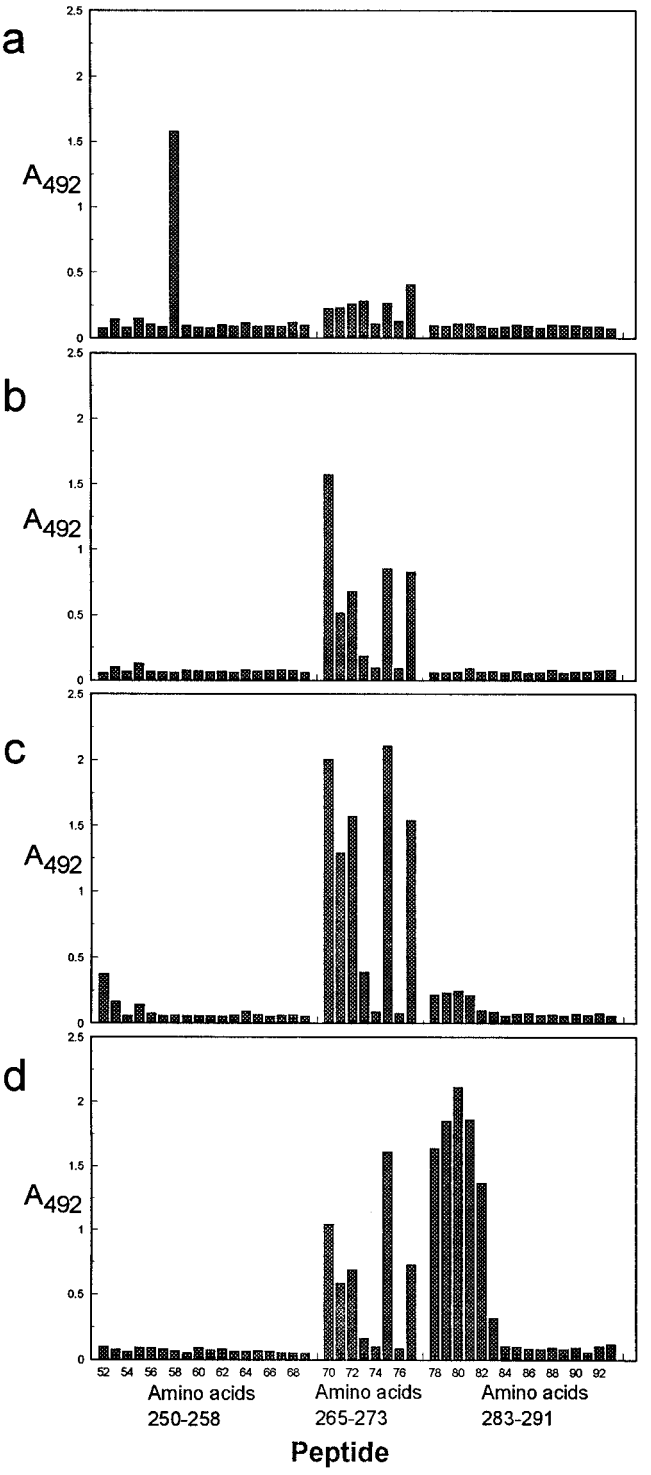

Fig. 3. Reactions of baby sera in ELISA tests with series of 9mer peptides listed in Table I. **a:** Baby 706 convalescent serum; **b:** baby 25 convalescent serum; **c:** baby 47 convalescent serum; **d:** baby 31 convalescent serum.

within the peptides but rather that there may be more than one antigenic site or several overlapping epitopes as has been described for influenza virus haemagglutinin [Schoofs et al., 1988]. Previous workers concluded that the carboxy-terminal part of the G protein was not immunogenic because they did not observe serological reactions using peptides based on the sequence of prototype strains [Norrby et al., 1987; Langedijk et al., 1996]. The high degree of amino acid variability observed in this region of the G protein and the demonstration that primary sera have variable reactions depending on the infecting virus are probably sufficient to account for the different results reported here. One possible alternative explanation is that the peptides used in this study were linked to pins, whereas the peptides used in the previous study [Norrby et al., 1987] were coated onto ELISA plates.

The biological activity of the antibody responses described above is not known, and some of the epitopes recognised by human sera in this study do not correspond with the amino acids where monoclonal antibody escape mutant changes occur. However, with respect to amino acids 283–291, one mouse monoclonal antibody was found to select a neutralisation escape mutant where the mutant change mapped to residue 287 [Martinez, 1996]. In this case the virus used for raising the monoclonal antibodies had a leucine residue in this position while the escape mutant had a proline residue, i.e., the exact reverse of what was observed with baby 31's serum. Thus neutralisation escape has been show to occur in vitro by varying the amino acids in the same way that naturally occurring virus isolates vary, indicating that antibody could be selecting these naturally occurring variants.

All the peptides recognised by the convalescent baby sera include potential N-glycosylation sites in at least some isolates though frequently not in the peptide sequence that is actually recognised (see Fig. 1). For example, peptide 7 includes the sequences NTT (RSB89-6256) or NIT (RSB89-6190), and this potential N-glycosylation site is conserved in most but not all isolates examined [Cane et al., 1991; Garcia et al., 1994; unpublished data]. The 17–18 pair of peptides include an N-glycosylation site in RSB89-6190 but not in RSB89-6256, and in this small study, it was only babies that had been infected with virus that was non-glycosylated at this site that showed reactions with this peptide. Only one baby's serum was observed to react strongly with peptides 23 and 24. With respect to the strains on which the peptides were based, there is not a glycosylation site in this area. However, one isolate from 1992 does have a potential N-glycosylation site here,^{285}NLS^{287} [Cane and Pringle, 1995] and recent isolates (epidemic 1994–95) from the Birmingham, UK, area likewise show the same potential N-glycosylation site (unpublished data). Both peptides 12 and 13 include two overlapping N-glycosylation sites that are variably present in isolates. The only area in this carboxy-terminal region of the G protein that has a potential N-glycosylation site to which no reaction was detected was a site present in the terminal 5 amino acids of some isolates.

The presence of potential N-glycosylation sites on the G protein is highly variable between RSV isolates [Cane et al., 1991; Sullender et al., 1991; Garcia et al., 1994]. The observation that linear epitopes recognised by primary infection sera are concentrated in the potential areas of N-glycosylation probably reflects their position on the outer surface of the molecule and suggests a possible mechanism for the virus to evade the immune response, namely, by modulating the glycosylation sites. It has been observed for influenza virus that carbohydrate can inhibit recognition by a monoclonal antibody [Skehel et al., 1984], and that the presence of N-glycans can interfere with the binding of neutralising antibodies to HIV [Back et al., 1994]. Also, it has been shown recently that recognition of RSV G protein by murine Mabs can be affected by glycosylation differences due to the cell type that the virus has been grown in [Garcia-Beato et al., 1996]. However, it has yet to be demonstrated that antibodies detected in this report are either neutralising in vitro or protective in vivo and the additional effects of O-glycosylation remain to be determined.

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